What Can Microdialysis Tell Us About the Temporal and Spatial Generation of Cytokines in Allergen-Induced Responses in Human Skin *In Vivo*?

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This study examined the suitability of microdialysis to assess the time course of cytokine generation from discrete sites within the skin following intradermal injection of allergen. Cytokines were recovered using two microdialysis probes, one close to the point of allergen injection and the other 1 cm away but within the area of the late-phase induration. Skin biopsies taken at both sites were stained immunocytochemically to investigate possible relationships between cytokine generation, expression of adhesion molecules, and recruitment of neutrophils and eosinophils during the late-phase allergic response. The cytokine response to probe insertion was assessed using a single probe in the opposite arm (control). At baseline, microdialysate contained low levels of IL-1α, IL-5, IL-8, IL-12, GM-CSF, and TNFα ($n = 27$–33). At control sites, this was followed by increases in IL-6 and IL-8 at 3 and 6 hours. Allergen increased TNFα levels in 3/11 individuals within 30 minutes at the injection site. Levels of IL-6 and IL-8 rose rapidly and were significantly greater ($P < 0.05$) than that of controls at 3 and 6 hours at both injection and distant sites. Adhesion molecule expression and leukocyte infiltration were elevated only at the allergen injection site, suggesting a complex relationship between cytokine generation and cellular events in allergic inflammation. In conclusion, microdialysis can be used to distinguish temporal and spatial changes in protein profiles in the skin. Furthermore, when used in conjunction with skin biopsies, it provides novel information about the mechanisms of dermal inflammation.

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INTRODUCTION

The ability to control inflammation, modulate immunological diseases, or accelerate tissue repair remains elusive largely because of the complexity of cytokine networks involved in their etiology. Great advances in understanding cytokine networks in disease have been made by studying biopsies, using isolated cells *in vitro*, and developing murine models of human disease (Izuhara, 2003; Gutermuth *et al.*, 2004; Pivarcsi and Homey, 2005; Hennino *et al.*, 2006). However, in order to understand more clearly the mechanisms underlying the initiation and development of human diseases at a local level, it is necessary to be able to gain an insight into both the temporal and the spatial generation of cytokines.

Many methods have been used to sample body fluids in order to measure the mediators generated from local inflammatory responses in the skin. Perhaps the easiest method is to take a blood sample. While this may be useful to measure the time course of the release of mediators, it gives little information about their source. Also, it may grossly underestimate mediator levels coming from a local inflammatory reaction (Heavey *et al.*, 1984; Petersen *et al.*, 1997a), while overestimating skin levels of mediators generated during a systemic response (Nedrebo *et al.*, 2004). At the opposite end of the scale, cytokines have been successfully measured in the extracellular fluid extracted from biopsies of rat skin by centrifugation (Nedrebo *et al.*, 2004). While this technique allows the measurement of cytokines at discrete sites, it may not be ethically acceptable in human volunteers because, to establish a time course of cytokine generation, multiple biopsies are necessary, each one of which leaves a permanent scar.

The method most often used to measure the local generation of cytokines in humans is the raising of skin blisters (Zweiman *et al.*, 1997; Teunissen *et al.*, 2002). However, Zweiman *et al.* (1997) reported that the trauma of raising the blister and the fitting of a skin chamber stimulated the generation of high levels of IL-6 and IL-8 with lower levels of RANTES and IL-1β in fluid sampled at 5 hours. Furthermore, this technique cannot be used in damaged or abraded skin.

Microdialysis is a well-established technique for the continuous sampling of small, water-soluble molecules within the extracellular fluid space *in vivo*. It has the advantage over other sampling techniques in that it can be used in intact tissues to follow temporal variations in the generation and the release of substances at discreet locations

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within the tissue space. Since its initial use in the brain of experimental animals for the recovery of neurotransmitters and other small hydrophilic solutes, microdialysis has been adapted for use in many other tissues including skin, adipose tissue, muscle, and the gastrointestinal tract (Clough, 2005). The recent development of large pore membranes (molecular mass cutoff >100,000 Da) has facilitated the use of microdialysis in the recovery of larger proteins and macromolecules from the tissue space (Sjogren *et al.*, 2002; Winter *et al.*, 2002, 2004; Averbeck *et al.*, 2006).

In our study, we have used the allergen-induced wheal-and-flare response as a model to explore the ability of microdialysis to detect temporal and spatial changes in cytokine generation and investigate possible relationships between cytokine generation and the expression of adhesion molecules and the recruitment of neutrophils and eosinophils. By inserting a separate probe into the opposite arm, the generation of cytokines in response to dermal injury caused by probe insertion was also investigated.

RESULTS

Macroscopical events in the skin

All participants developed a transient edema at the sites of implantation of microdialysis probes, which regressed within $1\frac{1}{2}$ hours. The injection of saline produced a small wheal, which resolved within 15 minutes.

Allergen injection stimulated the development of wheal-and-flare responses, the mean areas of which were 1.42 ± 0.14 and $24.33 \pm 2.86\ cm^2$ (mean ± SEM) respectively at 15 minutes after challenge. This was followed by a late-phase response that became macroscopically obvious from around 3 hours and had a mean area of $40.4 \pm 5.2\ cm^2$ at 6 hours after allergen challenge (Figure 1).

Cytokines

The release of selected cytokines in individual subjects is shown in Figure 2 and the mean levels ± SEM in Table 1.

Cytokine response to dialysis probe insertion. Microdialysis fluid was collected between 1 and $1\frac{1}{2}$ hours after probe insertion for measurement of "baseline" cytokine levels. The mean cytokine levels ± SEM (pg/ml) recovered from all three probes in all 11 volunteers were as follows: IL-1α 12.9 ± 6.0; IL-8 5.0 ± 1.4; IL-12 5.8 ± 2.3; GM-CSF 5.6 ± 2.2; and TNFα 7.4 ± 3.1 (all $n = 33$). IL-5 at baseline was 10.3 ± 2.4 pg/ml (a total of 27 probes in 9 volunteers). IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, and IFNγ were all below the level of detection of the assay.

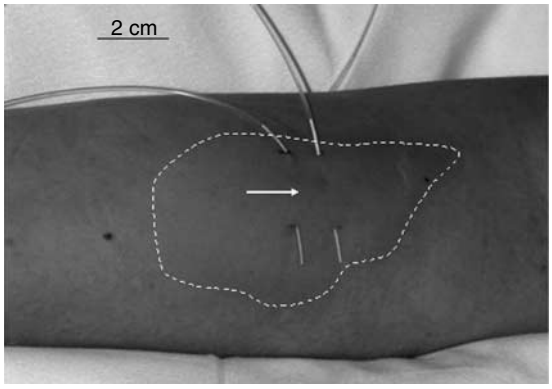

Figure 1. Photograph of a late-phase response at 6 hours after allergen challenge. The arrow indicates the site of the allergen injection, close to and halfway along the left-hand fiber, and the dotted line outlines the area of the late-phase response.

Control site. The levels of all cytokines recovered during the 30-minute period after injection of saline were not significantly different from baseline levels.

The levels of IL-1α recovered at both 3 and 6 hours were almost 50 pg/ml, significantly ($P = 0.02$) higher than those recovered at baseline (Table 1 and Figure 2). In contrast, the concentration of IL-1β recovered at 3 hours was below the level of detection, and only a small, but statistically significant ($P < 0.02$) increase was detected at 6 hours.

The largest cytokine increases seen at 3 and 6 hours after probe insertion were in IL-6 and IL-8 (Table 1 and Figure 2). IL-6 appeared to reach a maximum at 3 hours, with no significant difference ($P = 0.69$) between the mean levels detected at 3 and 6 hours. In contrast, the mean level of IL-8 recovered at 6 hours was significantly ($P = 0.004$) greater than that recovered at 3 hours. There were statistically significant correlations between IL-6 and IL-8 levels detected at both 3 hours ($P = 0.008$) and 6 hours ($P = 0.006$).

No significant increases in the levels of any other cytokine were seen at any time.

Allergen challenge site. The levels of IL-1α recovered at both 3 and 6 hours, but not those recovered during the 30-minute period after injection of allergen, were significantly higher than those recovered at baseline (Table 1 and Figure 2). Although the levels of IL-1α at 3 and 6 hours at the challenge site were numerically higher than those at the control site at the same times, the difference was not statistically significant as it was mainly due to a large response in one individual (Figure 2).

With IL-1β, there were small but significant increases in cytokine recovery at 3 and 6 hours. There were no significant differences between levels of IL-1β recovered at the control and allergen challenge sites.

There were significant increases above baseline in the levels of IL-6 at 30 minutes, 3 hours, and 6 hours, and of IL-8 at 3 and 6 hours (Table 1 and Figure 2). These increases were significantly greater ($P < 0.05$ at all times) than those seen at the control site. The level of IL-8, but not IL-6, was significantly ($P = 0.036$) higher at 6 hours than 3 hours. There were statistically significant correlations between IL-6 and IL-8 levels detected at both 3 hours ($P = 0.013$) and 6 hours ($P < 0.001$). There was no significant correlation between the levels of either cytokine and the area of the late-phase response.

The levels of TNFα recovered at the site of allergen challenge deserve special mention. Three individuals had significantly higher baseline levels of TNFα than the other eight (19.6 ± 2.1 *vs* 1.1 ± 0.8 pg/ml, $P = 0.015$). In these three individuals, the level of TNFα rose to 58.7 ± 6.6 pg/ml ($P = 0.045$ *vs* baseline) but had returned to baseline levels

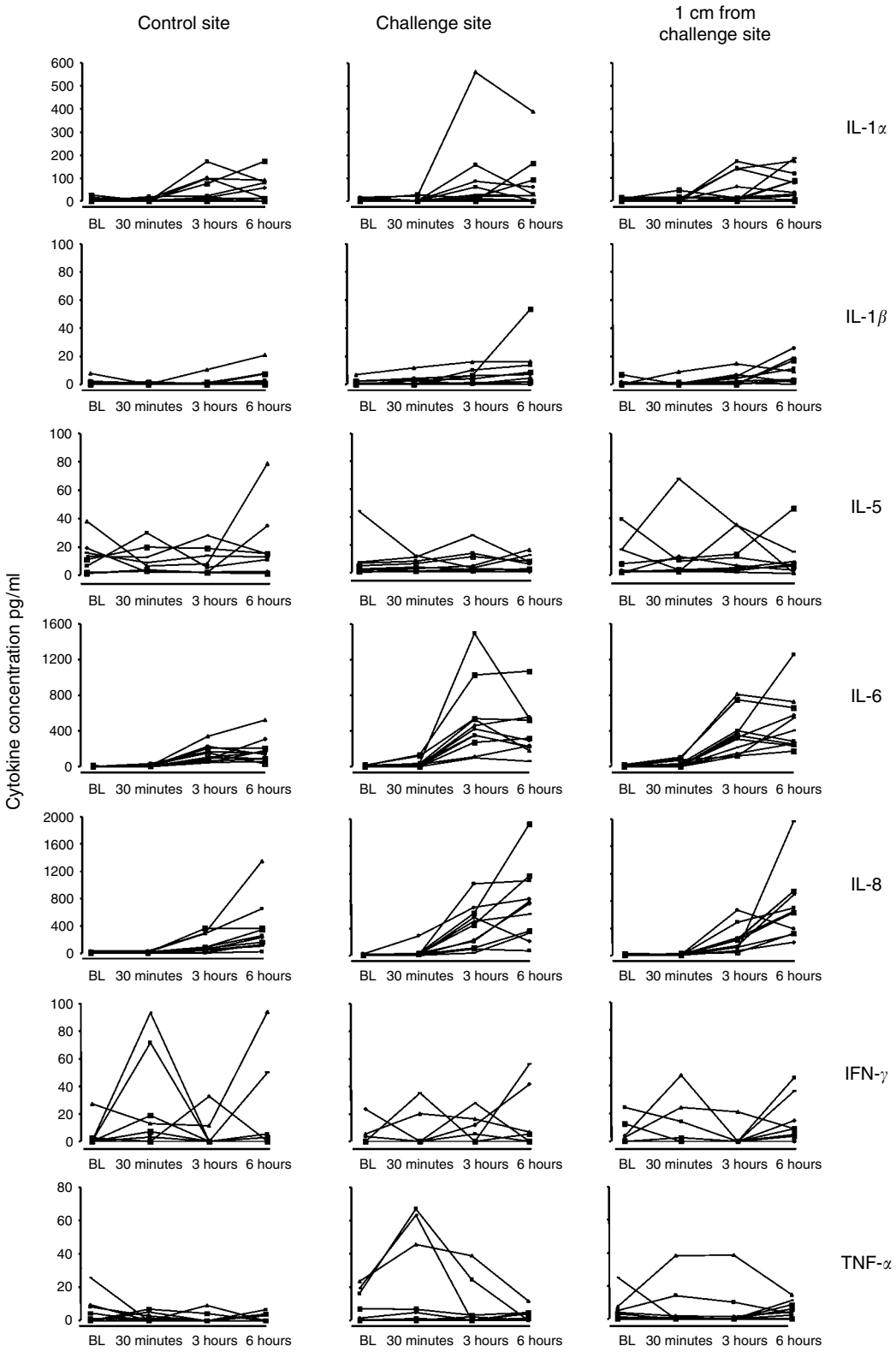

Figure 2. Graphs show cytokine recovery from individual volunteers. Data are from three sites: ''Control'', probe insertion site in the arm opposite to that of allergen challenge; ''Challenge site'', the site of allergen challenge; and ''1 cm from challenge'', the site 1 cm away from the allergen injection. The periods of dialysate collection were ''Baseline'', the 30 minutes period immediately before injection of allergen or saline (control); ''30 minutes'', the 30-minute period immediately following injection of allergen or saline; ''3 hours'', 3–3$\frac{1}{2}$ hours after injection of allergen or saline; and ''6 hours'', 6–6$\frac{1}{2}$ hours after injection of allergen or saline.

Table 1. Mean (±SEM) cytokine levels recovered from the skin by microdialysis

Cytokine	Baseline	30 minutes	3 hours	6 hours
IL-1α				
Control	8.8±3.3	3.3±1.8 NS	47.6±17.0 *P*=0.02	47.6±16.7 *P*=0.02
Challenge	6.0±2.1	4.9±2.9 NS	86.3±49.5 *P*=0.004	71.6±35.2 *P*=0.02
1 cm away	7.6±1.8	8.7±4.3 NS	51.9±20.2 NS	75.4±19.3 *P*=0.004
IL-1β				
Control	1.2±0.7	0.5±0.2 NS	1.3±0.9 NS	4.6±1.9 *P*=0.02
Challenge	1.2±0.6	2.0±1.1 NS.	4.1±1.6 *P*=0.008	9.8±4.7 *P*=0.008
1 cm away	1.0±0.6	1.1±0.8 NS	3.2±1.3 NS	8.7± 2.6 *P*=0.002
IL-6				
Control	2.0±1.0	7.0±2.7 NS	137.1±27.1 *P*=0.001	174.2±42.6 *P*=0.001
Challenge	3.7±1.6	37.2±13.4 *P*=0.006	513.6±124.1 *P*=0.001	381.6±84.1 *P*=0.001
1 cm away	3.3±1.8	31.4±11.5 *P*=0.006	354.9±70.1 *P*=0.001	485.6±95.4 *P*=0.001
IL-8				
Control	5.4±2.8	7.6±2.7 NS	116.9±38.2 *P*=0.002	333.9±112.9 *P*=0.001
Challenge	4.6±2.1	35.8±24.7 NS	408.4±93.7 *P*=0.001	732.4±93.6 *P*=0.001
1 cm away	4.9±2.3	7.5±2.7 NS	227.5±84.1 *P*=0.001	713.9±147.4 *P*=0.001

NS, nonsignificant; SEM, standard error of the mean.
Data are from three sites: ''Control''', probe insertion in the arm opposite to that of allergen challenge; ''Challenge''', the site of allergen challenge; and ''1 cm away'', the site 1 cm away from the allergen challenge site. The periods of dialysate collection were ''Baseline'', the 30-minute period immediately before injection of allergen or saline (control); ''30 minutes'', the 30-minute period immediately following injection of allergen or saline; ''3 hours'', 3–3½ hours after injection of allergen or saline; and ''6 hours'', 6–6½ hours after injection of allergen or saline. *P*-values indicate statistical significance of differences from baseline measurements.

by 3 hours (Figure 2). No rise in TNFα was detected in the other eight individuals.

There were no significant allergen-induced increases in IL-2, IL-4, IL-7, IL-10, IL-12, IFNγ, and GM-CSF up to 6 hours after challenge. Of the Th2 cytokines, IL-4 remained below the level of detection at all times, while IL-5, although detectable at all times, did not rise. At the allergen injection site, the mean level of IL-5 was 8.25±4.57 pg/ml at baseline and 6.85±1.76 pg/ml at 6 hours.

Site 1 cm from the allergen challenge site. At the site 1 cm away from allergen challenge, there was a variable elevation in IL-1α levels (Figure 2), the mean levels not being statistically significantly different from baseline at 30 minutes or 3 hours. At 6 hours, IL-1α levels were statistically significantly ($P=0.004$) higher than baseline values but not statistically significantly different from either the control or allergen challenge sites at the same time.

Similar to the allergen challenge site, there were significant increases of IL-6 above baseline at 30 minutes, 3 hours, and 6 hours, and of IL-8 at 3 and 6 hours at the site 1 cm away from the challenge site (Table 1 and Figure 2). Again, the level of IL-8, but not IL-6, was significantly ($P=0.016$) higher at 6 hours than 3 hours. Unlike at the challenge site, there were no statistically significant correlations between IL-6 and IL-8 levels detected at either 3 hours ($P=0.405$) or 6 hours ($P=0.728$).

TNFα levels remained close to baseline at all times as did all the other cytokines assayed.

Adhesion molecules and cellular influx

The expression of adhesion molecules and cellular influx was assessed 6 hours after allergen injection in biopsies from six different volunteers. The expression of all adhesion proteins (Figure 3) was significantly higher at the site of injection compared with that 1 cm away, the respective numbers of positive cells/mm^2 being 14.2 ± 3.3 and 2.4 ± 1.7 ($P = 0.02$) for E-selectin, 17.02 ± 3.3 and 3.4 ± 1.4 ($P = 0.025$) for ICAM-1, and 3.0 ± 0.8 and 0.2 ± 0.1 ($P = 0.016$) for VCAM-1.

The influx of neutrophils and eosinophils was assessed 6 hours after allergen injection in the same biopsies used for the expression of adhesion proteins (Figure 4). The numbers of both cells were significantly higher at the site of injection compared with that 1 cm away, the respective numbers of positive cells/mm^2 being 495 ± 50 and 7.6 ± 3.0 ($P < 0.0001$) for neutrophils and 42.9 ± 17.7 and 11.0 ± 7.9 ($P = 0.044$) for eosinophils.

DISCUSSION

In this study, we have used microdialysis and multiple cytokine protein array analysis to assess cytokine generation in human skin *in vivo* in real time. By using intradermal allergen challenge as a model, we have shown that microdialysis is able to detect temporal and spatial changes in cytokine generation over and above those generated in response to wounding caused by probe insertion. Also, by comparing cytokine levels at 6 hours with microscopical assessment of biopsies taken at the same time, we have shown that it is possible to explore the relationships between cytokine generation and the expression of adhesion molecules and the recruitment of neutrophils and eosinophils.

While dermal microdialysis has been shown to recover small hydrophilic molecules, such as histamine and nitric oxide (Petersen *et al.*, 1995; Clough *et al.*, 2002), and products of metabolism (Muller, 2002) with good efficiency,

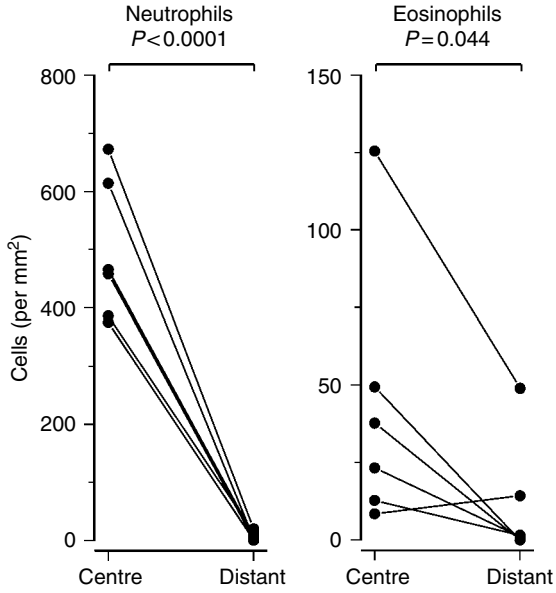

Figure 4. Influx of neutrophils and eosinophils. The number of nucleated cells staining positive for EG2 (eosinophils) or neutrophil elastase (neutrophils) 6 hours after allergen challenge was assessed in biopsies from six volunteers. Biopsies were taken from the allergen challenge site (center) and 1 cm away from the allergen challenge site.

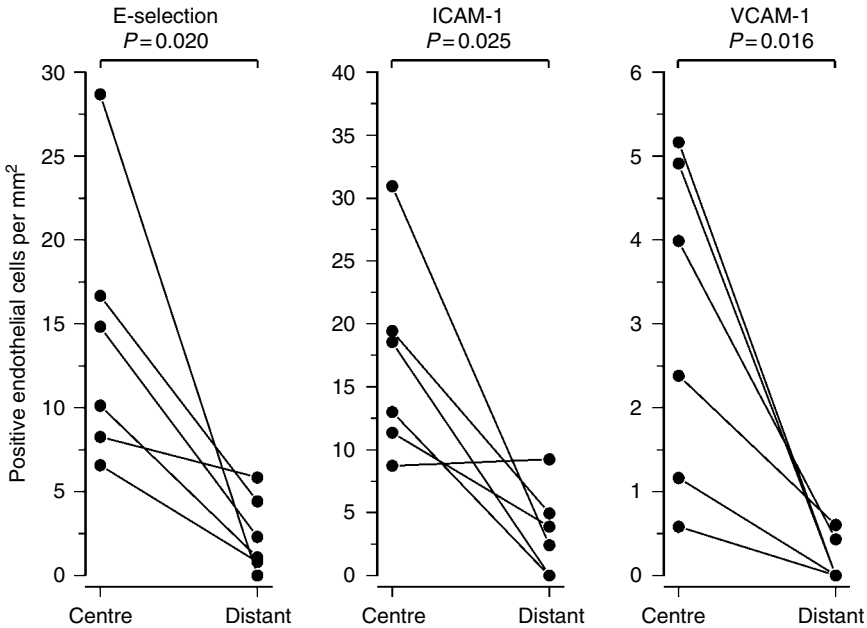

Figure 3. Expression of adhesion molecules. The number of nucleated blood vessel endothelial cells staining positive for E-selectin, ICAM-1, and VCAM-1 6 hours after allergen challenge was assessed in biopsies from six volunteers. Biopsies were taken from the allergen challenge site (center) and 1 cm away from the allergen challenge site.

its ability to recover large relatively hydrophobic molecules, such as cytokines which may adhere to tissue elements surrounding the probe and/or to the probe membrane, is much reduced (Ao *et al.*, 2004). Using a commercial membrane with a molecular cutoff of 100,000 Da, the recovery of a number of cytokines, including IL-2, IL-4, IL-5, IL-6, IFN-γ, and TNFα, has been reported to be as low as 1% (Sjogren *et al.*, 2002; Schutte *et al.*, 2004; Waelgaard *et al.*, 2006). Recovery *in vitro* with dialysis probes having a membrane with a molecular cutoff of 3,000,000 Da, as used in our experiments, is higher, being 45% for IL-6, 28% for IL-1α, 22% for nerve growth factor, and 18% for human serum albumin (Winter *et al.*, 2002).

A second factor that must be taken into consideration with dermal microdialysis is the poor diffusibility of small molecules such as histamine and sodium fluoride in the skin, the maximum lateral diffusion of substances being around 2 mm (Petersen *et al.*, 1997b; Clough *et al.*, 2002). While this may be an advantage when investigating the localization of released substances, it also means that prolonged dialysis leads to a partial depletion of dialyzable molecules in the immediate vicinity of the probes (Clough *et al.*, 2002). Thus, although it is scientifically legitimate for us to cite only the cytokine levels present in the dialysate at any time, local extracellular concentrations are likely to be considerably greater.

Before discussing the effects of allergen challenge, it is pertinent to discuss the stimulation of cytokine generation in response to trauma caused by probe insertion. As would be expected, levels of all cytokines were low or undetectable 1 hour after probe insertion. The observation that trauma stimulated the generation of IL-6 and IL-8 extends previous microdialysis and skin blister studies respectively (Sjogren *et al.*, 2002; Zweiman *et al.*, 1997; Nocker *et al.*, 1999; Averbeck *et al.*, 2006).

Following allergen injection, the levels of IL-6 and IL-8 recovered from the challenge site rose rapidly, reaching levels significantly higher than those at the control site. These data together with *in vitro* and *in vivo* studies in the airways (Teran *et al.*, 1996) confirm that IL-6 and IL-8 are generated following allergen challenge. The observation that IL-6 levels peaked by 3 hours agrees with the skin blister study of Lee *et al.* (1992), who reported rapid generation of IL-6 with a maximum at $3\frac{1}{2}$ hours after allergen challenge. As seen at the control site, levels of IL-8 rose more slowly than those of IL-6, not achieving statistical significance above baseline levels until 3 hours and still rising at 6 hours. The observation that the production of IL-8 was significantly increased by allergen challenge agrees with the data of Zweiman *et al.* (1997) using the skin window technique.

Increased levels of TNFα were found in the first 30-minute collection period at the allergen challenge site only. The early timing of the TNFα peak would support the suggestion of the release of pre-formed TNFα from mast cells (Bradding *et al.*, 1995; Coward *et al.*, 2002). Detectable increases of TNFα were found in only 3/11 volunteers. Interestingly, in their skin chamber study, Zweiman *et al.* (1997) found increased TNFα in only 3/15 individuals following allergen challenge. This suggests a great variation between individuals in the generation of TNFα, which, in our study, did not correlate with the production of other cytokines or the severity of other features of the late-phase response.

Interestingly, levels of IL-6 and IL-8 were also increased above control at the site 1 cm away from allergen challenge. In contrast, no increased levels of TNFα were detectable at this site. Thus, it is unlikely that increased levels of IL-6 and IL-8 at the distant site were due to diffusion of the cytokines. It is also unlikely to be a chemical signal diffusing through the dermis or being carried by the blood or the lymphatics because of their architectural arrangement. Therefore, it is proposed that cytokines must be actively generated over a wider area than the point of allergen injection. But how does the message to generate cytokines get to the cells 1 cm away from the site of challenge? There are two other more obvious possibilities. The first is that cytokine secretion was stimulated by the release of neuropeptides, particularly calcitonin gene-related peptide (Schmelz *et al.*, 1997). This is supported by *in vitro* evidence that calcitonin gene-related peptide stimulates the production of IL-6 in human fibroblasts (Sakuta *et al.*, 1995) and peripheral blood mononuclear cells (Cuesta *et al.*, 2002) and IL-8 in human synovial fibroblasts (Raap *et al.*, 2000) and monocytes (He *et al.*, 2002). The second possibility is that of conducted responses involving the generation of electrical currents and Ca^{2+} signalling across gap junctions of the arteriolar walls. Such mechanisms have been previously shown in the regulation of vasomotor tone (Dora *et al.*, 2003).

In vitro studies have identified a wide spectrum of pro-inflammatory cytokines to be released following allergen challenge including a group of TH2-lymphocyte-associated cytokines, IL-4, IL-5, and IL-13, which promote the development of allergic response. Supporting evidence of their involvement in allergy *in vivo* comes primarily from their demonstration in bronchoalveolar lavage fluid (Broide and Firestein, 1991; Virchow *et al.* 1995; Kroegel *et al.*, 1996; Teran *et al.*, 1999; Batra *et al.*, 2004; Julius *et al.*, 2004). In the skin, large numbers of cells both expressing IL-4 and IL-5 mRNA and staining for cytokine protein have been reported to be present at the site of allergen-induced cutaneous late-phase reactions in atopic subjects at 6 hours (Barata *et al.*, 1998). Consequently, we were a little surprised not to recover increased amounts of either. There are several possible explanations for this. First, IL-4 in particular is produced in low levels and is not always detectable following allergen challenge of the airways (Virchow *et al.* 1995). Second, the 6-hour time point is too early for the generation of IL-4 and IL-5. The majority of bronchial challenge studies have measured cytokine generation 18 hours or later after allergen challenge (Broide and Firestein, 1991; Virchow *et al.* 1995; Kroegel *et al.*, 1996; Teran *et al.*, 1999; Batra *et al.*, 2004; Julius *et al.*, 2004). However, in one study, a small increase in IL-5 levels was detected in bronchoalveolar lavage at 4 hours (Teran *et al.*, 1999). Third, these cytokines remain cell associated so that they cannot be recovered by dialysis in the skin.

At the site of allergen injection, biopsies taken at 6 hours showed marked increases in the adhesion molecules

E-selectin, ICAM-1, and VCAM-1. Also, there was a 65-fold increase in neutrophils and a 4-fold increase in eosinophils, consistent with previous studies (Barata *et al.*, 1998; Litchfield *et al.*, 1996). However, at the site 1 cm away from allergen injection, there was little staining for adhesion molecules and no inflammatory cell accumulation. This was somewhat of a surprise considering that the levels of IL-6 and IL-8, both of which have been reported to increase adhesion molecule expression and inflammatory cell accumulation (Borish *et al.*, 1989; Douglass *et al.*, 1996), were similar to those recovered from the site of allergen injection. Further, while endothelial cells are good producers of the NF-κB-stimulated cytokines, IL-6 and IL-8, the generation of these cytokines is often accompanied by other NF-κB-stimulated products, including the adhesion molecules ICAM-1 and VCAM-1 (Lin *et al.*, 2005). A possible suggestion to explain this apparent anomaly is that IL-6 and IL-8 were generated from non-vascular tissues such as fibroblasts (Kubo and Kuroyanagi, 2005) rather than from endothelial cells.

In conclusion, we have shown that microdialysis in combination with multiplex cytokine array assays can be used to distinguish temporal and spatial changes in protein profiles in the skin following both wounding and the development of allergic inflammation. The observation that adhesion molecule expression and leukocyte infiltration were elevated at the site of allergen injection only suggests a complex relationship between cytokine generation and the cellular events occurring in allergic inflammation. Furthermore, when used in conjunction with other tissue-sampling techniques, such as immunohistochemical analysis of skin biopsies, microdialysis may be used to provide novel information about disease mechanisms in the skin.

MATERIALS AND METHODS

Volunteers

Seventeen healthy, normotensive volunteers (15 male and 2 female; age range 20–30 years) were recruited into the study. All gave a positive skin prick test (weal of 5 mm in diameter at 10–15 minutes) to either 6-grass mix or *Dermatophagoides pteronyssinus* (house dust mite) (ALK, Horsholm, Denmark). The study was approved by the Southampton & South West Hampshire Research Ethics Committee (LREC 003/02) and conformed to the Declaration of Helsinki Principles. All volunteers gave written informed consent. The study was performed in the thermoneutral environment of the Wellcome Trust Clinical Research Facility of Southampton General Hospital.

Microdialysis

Two linear cutaneous microdialysis membranes (3,000 kDa molecular mass cutoff) were inserted 11 mm apart, into the volar surface of each forearm of the volunteers, under topical local anesthesia (EMLA cream, 2.5% prilocaine, 2.5% lidocaine, Astra Pharmaceuticals, Kings Langley, UK). Each membrane ran for a length of 20 mm, at a depth of approximately 0.7 mm. A 1-hour period was allowed for recovery from local anesthesia and trauma before the start of perfusion with Ringer's solution at a rate of 5 μl/min using a microinfusion pump. Dialysate samples were collected in preweighed vials and stored at –80°C before analysis. To assess baseline cytokine levels, dialysate was collected from each probe for 30 minutes before the intradermal injection in one arm of Timothy Grass or *D. pteronyssinus* extract (300 SQ units in 20 μl Ringer's solution). The injection was made 1 mm from and parallel to the microdialysis fiber nearer to the wrist and 10 mm from the fiber further up the arm. An injection of the same volume of Ringer's solution ($n = 8$) or no injection ($n = 3$) at the site on the contralateral forearm served as a negative control site. Dialysate was collected at timed intervals, 0–30 minutes (early), 3–3½ hours (mid), and 6–6½ hours (late) after intradermal injection. Probe perfusion was stopped between collections.

Area of late-phase responses

The mean area of the late-phase response was measured by planimetry 6 hours after allergen challenge.

Cytokine analysis

Dialysate cytokine content was assayed using a 16-well human cytokine assay (Novagen Proteoplex) for IL-1α, 1β, 2, 4, 6, 7, 8, 10, 12, GM-CSF, IFNγ, and TNFα. Dialysate samples were diluted 1 in 2 using the standard diluent provided. A four-point standard curve was constructed at levels of 800, 400, 15, and 0 pg/ml. The detection level was 5 pg/ml. IL-5 was assayed in undiluted dialysate samples from nine volunteers using a separate enzyme immunometric assay (TiterZyme) with a detection level of 3 pg/ml.

Determination of adhesion molecule expression and cellular infiltration

Six hours after challenge, the area around the allergen injection and an area 1 cm away but within the late-phase response induration were infiltrated with 2% w/v lignocaine local anesthetic and then 3 mm punch biopsies were taken (Steifel Laboratories, Woburn Green, UK). Each biopsy was divided into three, in order to obtain pieces small enough for the satisfactory infiltration of fixative, and fixed overnight in ice-cold acetone containing the enzyme inhibitors iodoacetamide (20 mM) and phenyl methyl sulfonyl fluoride (PMSF, 2 mM). Specimens were then embedded in glycolmethacrylate (JB4 kit, Park Science Ltd, Northants, UK) as described previously (Church *et al.*, 2002). Sections of 2 μm thickness were cut and stained immunocytochemically using biotinylated mouse anti-mouse F(ab)2 (Dakopatts) as the secondary antibody (Church *et al.*, 2002). The following primary antibodies were used: E-selectin (Chemicon, Temecula, CA); ICAM-1 (BioSource International, Nivelles, Belgium); VCAM-1 (Serotec, Kidlington, UK); neutrophils (anti-neutrophil elastase, DAKO Ltd, Cambridge, UK); and eosinophils, EG2 (Diagnostics Development, Uppsala, Sweden). Immunocytochemically positive nucleated cells were counted in two high-power fields and the average number was expressed as cells/mm^2.

Statistical analyses

Results are expressed as mean ± SEM. As it was not possible to assume Gaussian distributions of the data, differences between cytokine recoveries from the same dialysis probes at different times were assessed by the Wilcoxon matched-pairs test and differences between cytokine recoveries from dialysis probes at different sites by the Mann–Whitney *U*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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